

# Structural determinants of the selectivity of KTS-disintegrins for the $\alpha 1\beta 1$ integrin

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**Abstract** KTS-disintegrins are a subfamily of short monomeric disintegrins that are potent and selective inhibitors of  $\alpha 1\beta 1$  integrin. The amino acid sequence of the new KTS-disintegrin, viperistatin, differs from previously characterized obtustatin in three residues at position 24 (within the integrin binding loop), 38 (hydrophobic core) and 40 (C-terminal region). Noteworthy, viperistatin is about 25-fold more potent than obtustatin inhibiting the binding of this integrin to collagen IV. Synthetic peptides representing the full-length of integrin-binding loops of these disintegrins showed that the Leu24/Arg substitution appears to be partly responsible for the increased inhibitory activity of viperistatin over obtustatin.

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**Keywords:** Disintegrin; Integrin antagonist; Collagen receptor; KTS-peptide; Cell adhesion

## 1. Introduction

The collagen receptors create the separate subclass of integrins that specifically interact with different types of collagens [1,2]. Among them, the most characterized are  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins, which belong to I-domain containing integrins. I-domain is a region within  $\alpha$ -subunit containing approximately 200 amino acids, which has been identified as a specific interactive part responsible for the ligand binding [3,4]. Both integrins are expressed on various cell types and play significant roles in the physiology of many organs. Recently, they have become an interesting target for the development of new cancer therapy, supported by inhibition of angiogenesis.  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins are highly expressed on the microvascular endothelial cells, and blocking of their adhesive properties by

monoclonal antibodies [5,6] or snake venom disintegrins [7] significantly reduced vascularization ratio and tumor growth in animal models. Moreover, studies with  $\alpha 1$  knockout mice revealed major reduction of experimental tumor growth [8] and similar studies with  $\alpha 2$  knockout mice are on the way [9,10]. Although  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  are structurally very homologous, their biological interactions appear to be significantly different.  $\alpha 1\beta 1$  integrin is a selective receptor of basement membrane collagen type IV, whereas  $\alpha 2\beta 1$  is highly specific for fibrillar collagens type I–III. Moreover, studies with genetic substitution of cytoplasmic domains of  $\alpha 1$  and  $\alpha 2$  subunits in transfected human mammary epithelial cells revealed involvement of these two integrins in different signal transduction pathways [11].  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins showed also different patterns of interaction with snake venom-derived proteins.  $\alpha 1\beta 1$  integrin specifically interacts with disintegrin obtustatin [7,12], whereas  $\alpha 2\beta 1$  showed potent binding to C-lectin-type proteins [13,14].

Snake venom disintegrins are a family of low molecular weight proteins, showing anti-adhesive properties that are related to their specific interactions with certain integrins [15,16]. Structurally, they may occur as monomers or dimers and have the molecular weight in the range from 4 to 15 kDa. Snake venom disintegrins could be divided into three groups according to their functional interaction with specific integrin receptors. The first group includes disintegrins that interact with RGD-dependent integrins ( $\alpha II\beta 3$ ,  $\alpha v$ -integrins, and  $\alpha 5\beta 1$ ) and is mainly represented by the disintegrins that contain RGD sequence in their active site. The disintegrins containing sequences related to RGD such as KGD [17], MGD [18] or WGD [19] could be also assigned to this group. Second group is created by heterodimeric disintegrins having MLD sequence in the active site. They specifically block the function of certain leukocyte integrins, including  $\alpha 4$ -integrins and  $\alpha 9\beta 1$  [20,21]. The last functional group of disintegrins includes selective inhibitors of  $\alpha 1\beta 1$  integrin. Currently, it is represented by the only one disintegrin, obtustatin, that contains KTS sequence in its active site [7,12]. Structurally, obtustatin belongs to short monomeric disintegrins and recently its 3-D structure was composed based on the NMR coordinates [22]. In the presented work, we report another KTS-containing disintegrin, viperistatin, which also specifically interacts with  $\alpha 1\beta 1$  integrin.

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**Abbreviations:** BSA, bovine serum albumin; CMFDA, 5-chloromethylfluorescein diacetate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; PBS, phosphate-buffered saline

## 2. Materials and methods

### 2.1. Materials

Monoclonal antibody against 7S domain of collagen IV and purified human  $\alpha 1 \beta 1$  integrin were purchased from Chemicon (Temecula, CA). Highly purified fibrinogen was a gift from Dr. A. Budzynski (Temple University, Philadelphia, PA); recombinant human VCAM-1/Ig was received from Dr. R. Lobb (Biogen); human vitronectin was purchased from Chemicon; human fibronectin and laminin were purchased from Sigma (St. Louis, MO).

**Cell lines.** K562 cells transfected with  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 6$  integrins were provided by Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA); JY cells expressing  $\alpha v \beta 3$  were a gift from Dr. Burakoff (Dana Farber Cancer Institute, Boston, MA);  $\alpha 9$ -transfected SW480 cells were provided by Dr. Sheppard (University of California, San Francisco, CA); A5 cells, Chinese hamster ovary (CHO) cells transfected with human  $\alpha IIb \beta 3$  integrin [23] were kindly provided by Dr. M. Ginsberg (Scripps Research Institute, La Jolla, CA); and K562 and Jurkat cell lines were purchased from ATCC (Manassas, VA).

**Snake venoms.** Lyophilized *Vipera lebetina obtusa* and *Echis suchoreki* venoms were purchased commercially from Latoxan (France). Venom of *Vipera palestinae* was collected from life specimens and kept in captivity in the Zoology Department of Tel-Aviv University. The snakes were manually milked during a period of one year and the venom pooled from several snakes was lyophilized and kindly provided to us by Prof. Avner Bdolah.

### 2.2. Purification of collagen type IV

Collagen IV secreted by HT-1080 cells was purified from cell culture media. HT-1080 cells were seeded into three separate intercommunicating stacks of culture flasks (6000 cm<sup>2</sup> each; Cell Factory<sup>TM</sup>, Nunc). To harvest collagen IV, the cells were incubated in Dulbecco's modified Eagle's medium supplemented with L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako, Osaka, Japan) at a concentration of 40  $\mu$ g/ml without fetal bovine serum for 24 h on each of six successive days. The medium was filtered through a 1.6  $\mu$ m glass-fiber filter (Millipore) and supplemented with the following reagents at the indicated concentrations: 0.1 M Tris-HCl buffer, pH 7.4, 0.4 M NaCl, 25 mM EDTA, and 0.02% NaN<sub>3</sub>. High molecular weight proteins in the medium were concentrated approximately 10-fold at 4 °C. Proteins in the concentrated media were precipitated overnight at 4 °C with 175 mg/ml of ammonium sulfate and collected by centrifugation at 15000  $\times$  g for 1 h at 4 °C. The pellets were resuspended overnight in the storage buffer (0.1 M Tris-HCl buffer, pH 7.4, 0.4 M NaCl, 25 mM EDTA and 0.02% NaN<sub>3</sub>) and insoluble material was removed by centrifugation. The supernatant was dialyzed twice against 200 vol. of buffer I (2 M urea, 0.2 M NaCl, 5 mM EDTA and 0.02% NaN<sub>3</sub> in 0.1 M Tris-HCl buffer at pH 7.4), and the samples were chromatographed on a DEAE-cellulose column (1.5 cm  $\times$  10 cm) (DE 52, Whatman) equilibrated and eluted with buffer I, and the flow-through fraction was collected. The sample was dialyzed against 200 vol. of buffer II (2 M urea, 2 mM EDTA, and 0.02% NaN<sub>3</sub> in 0.075 M Tris-HCl buffer at pH 7.8) and chromatographed on the second DEAE-cellulose column. Collagen IV was collected in the flow-through fraction and loaded onto a column (1.5 cm  $\times$  2 cm) of Q-Sepharose (Amersham) in the same buffer. The purified collagen IV was dialyzed against storage buffer and concentrated on a membrane filter (YM-100, Millipore) under a pressure of 48 kPa.

### 2.3. Purification of disintegrins

Obtustatin, viperistatin and echistatin were purified from the venoms of *Vipera lebetina obtusa*, *Vipera palestinae*, and *Echis suchoreki*, respectively, using the previously described two-steps reverse-phase high-performance liquid chromatography (HPLC) [20,24]. Briefly, the venoms were dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to HPLC on a C<sub>18</sub> (250  $\times$  10 mm) reverse-phase column from Vydac (Hesperia, CA). The linear acetonitrile gradient (0–80% in 0.1% TFA over 45 min at a flow rate 2 ml/min) was applied for elution of venom fractions. Separation was monitored at 230 nm single wavelength by UV detector (HPLC system from Waters). Obtustatin and viperistatin were eluted with 48% and 40% of acetonitrile, respectively, whereas echistatin was eluted with about 30% of acetonitrile. Fractions containing the disintegrins were re-chromatographed using the same column and developed with a “flatter” acetonitrile gradient. The purity of the disintegrins was assessed by SDS-PAGE and matrix-assisted

laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Protein concentration was determined with the bicinchoninic acid protein quantification kit (Pierce Co.) with bovine serum albumin (BSA) as a standard.

### 2.4. Structural characterization of viperistatin

Purified viperistatin was reduced and alkylated as described previously [20]. *S*-Pyridylethylated (PE-) viperistatin was initially characterized by N-terminal sequencing (using either an Applied Biosystem Precise instrument), amino acid analysis (using a Beckman Gold Amino Acid Analyzer after sample hydrolysis with 6 N HCl for 24 h at 110 °C), and MALDI-TOF MS (as above). Quantitation of free cysteine residues and disulfide bonds was done as described [25]. The primary structure of viperistatin was determined by N-terminal sequence analysis of the PE-protein and of HPLC-purified endoproteinase Lys-C-derived overlapping peptides [26].

### 2.5. Peptide synthesis

Peptides were prepared by solid phase synthesis on Wang resin and ChemSpeed ASW 2000 synthesizer using Fmoc method and Benzo-triazole-1-yl-oxy-tris (dimethylamino)-phosphonium hexafluorophosphate as a coupling reagent. A pure peptide was obtained after implementation of triple coupling for each cycle. The peptide was cleaved from resin with 95% TFA and 1% triethylsilane and its structure was characterized by ESI-MS. The peptides were dried by lyophilization before application to adhesion assay.

### 2.6. Cell adhesion studies

Adhesion studies of cultured cells labeled with 5-chloromethylfluorescein diacetate (CMFDA) were performed as described previously [27].

### 2.7. ELISA assay

96-well plates (BD Falcon, Franklin Lakes, NJ) were coated with purified  $\alpha 1 \beta 1$  (5  $\mu$ g/ml) overnight at 4 °C in phosphate-buffered saline (PBS). The wells were blocked with 5% non-fat milk (BioRad, Hercules, CA) in PBS/Tween 20 (PBST) buffer. Increasing concentrations of disintegrins or synthetic peptides were added in Tris-HCl, pH 7.4, buffer containing 150 mM NaCl, 1 mM MnCl<sub>2</sub>, and 1% BSA, and the plate was incubated for 30 min at 37 °C. After washing with the same buffer, purified, human collagen IV was added at a concentration of 2  $\mu$ g/ml. Incubation was continued for another 30 min at 37 °C. The bound collagen IV was detected by incubation with monoclonal antibody against 7S domain of collagen IV, following goat anti-human IgG conjugated with alkaline phosphatase (Sigma) as described previously [28].

## 3. Results and discussion

The new KTS-containing disintegrin viperistatin was purified from the venom of *Vipera palestinae*. Mass spectrometric analysis of native and reduced *S*-pyridylethylated viperistatin yielded isotope-averaged molecular masses of 4454.5 and 5303.2 Da, respectively. Incubation of viperistatin with 4-vinylpyridine under denaturing, non-reducing conditions did not change its molecular mass. Thus, the mass difference of 848.7 clearly indicated that viperistatin contained eight cysteine residues engaged in the formation of 4 disulfide bonds. The complete primary structure of viperistatin was established by N-terminal sequencing of EP-protein (Fig. 1). Viperistatin appeared to be highly homologous to obtustatin, another KTS-containing disintegrin [12,13,22,29], differing from it in just three residues at positions 24, 38 and 40. Structurally, KTS-disintegrins belong to a family of short monomeric disintegrins, which is mainly represented by RGD-containing disintegrins such as echistatin, eristostatin, ocellatusin, multisquamatin, pyramidin and leucogastin [25,30–32]. However, significant differences have been observed in the primary structure of short monomeric KTS-disintegrins and

Viperistatin	1 5 10 15 20 25 30 35 40 45 50	CTTG <u>P</u> CC <u>R</u> Q <u>C</u> KLKPAGTT <u>C</u> W-- KTS <u>R</u> TSHY <u>C</u> TGKS <u>C</u> DC <u>P</u> Y <u>V</u> Q <u>G</u>
Obtustatin		CTTG <u>P</u> CC <u>R</u> Q <u>C</u> KLKPAGTT <u>C</u> W-- KTS <u>L</u> TSHY <u>C</u> TGKS <u>C</u> DC <u>P</u> L <u>V</u> Y <u>G</u>
Echistatin		ECESGP <u>C</u> CRN <u>C</u> FLKEGT <u>I</u> CKRA RGD DMDDY <u>C</u> NGKT <u>C</u> DC <u>P</u> RNPHKGPAT
Eristostatin		QE <u>E</u> PCATG <u>P</u> CC <u>R</u> R <u>C</u> FKFRAGKV <u>C</u> RV <u>A</u> RGD WNDDY <u>C</u> TGKS <u>C</u> DC <u>P</u> RN <u>P</u> WNG
Ocellatusin		DCESGP <u>C</u> CDN <u>C</u> FLKEGT <u>I</u> CKMA RGD NMHDY <u>C</u> NGKT <u>C</u> DC <u>P</u> RN <u>P</u> YKGEHDT
		* * * * * * * * * *

Fig. 1. Comparison of the amino acid sequences of the short, monomeric disintegrins: viperistatin (*V. palestinae*, this work), obtustatin (*V. lebetina obtusa* [12]), echistatin (*E. sochureki* [30]), eristostatin (*E. macmahoni* [31]) and ocellatusin (*E. ocellatus* [24]). Cysteines are underlined and the active motifs are in italics. The double underlined are amino acid residues that are different in viperistatin and obtustatin molecule. Asterisks below the multiple sequence alignment indicate residues that are absolutely conserved among short monomeric disintegrins.

RGD-disintegrins (Fig. 1). KTS-disintegrins contain lower molecular weight resulting in shorter polypeptide chains (41 amino acids vs. 48–50 amino acids). The major difference is the composition of integrin-binding loop. The active loop of KTS-disintegrins is shorter by two amino acids and does not contain any RGD related motif. This structural feature is strictly reflected in activity. As shown in Table 1, KTS-disintegrins do not express any anti-RGD-dependent integrins activity, whereas RGD-containing echistatin potently inhibits  $\alpha$ Ib $\beta$ 3,  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1 integrins in adhesion assay. On the other hand, obtustatin and viperistatin are potent inhibitors of  $\alpha$ 1 $\beta$ 1 integrin, and this kind of activity has not been found for any RGD-disintegrins. Moreover, both KTS-disintegrins did not show any activity against other integrins such as  $\alpha$ 4 $\beta$ 1,  $\alpha$ 6 $\beta$ 1, and  $\alpha$ 9 $\beta$ 1 (Table 1). Particularly interesting is the lack of interaction with  $\alpha$ 2 $\beta$ 1 integrin that is very homologous to  $\alpha$ 1 $\beta$ 1 integrin, including sharing of similar endogenous ligands.

Previously, we identified the active site of obtustatin as a KTS motif, using short peptide synthesis [7]. Viperistatin also contains the same motif in the related site and shows also anti- $\alpha$ 1 $\beta$ 1 integrin activity (Table 1, Fig. 2). However, viperistatin appeared to be a much potent inhibitor of  $\alpha$ 1 $\beta$ 1 integrin than obtustatin. In the adhesion assay, viperistatin was 25-fold more active in inhibition of  $\alpha$ 1K562 cell adhesion to both collagen IV and collagen I. The same trend was observed when the inhibitory activity of viperistatin and obtustatin was compared in ELISA, where binding of collagen type IV to immobilized purified  $\alpha$ 1 $\beta$ 1 integrin was investigated (Fig. 2A).

The fact that viperistatin and obtustatin differ in just three residues (L<sup>24</sup>/R within the integrin binding loop, L<sup>38</sup>/V in the hydrophobic core, and P<sup>40</sup>/Q in the C-terminal region) (Fig. 3), and viperistatin is a much potent inhibitor of  $\alpha$ 1 $\beta$ 1 integrin than obtustatin, prompted us to investigate their structure-function correlations using synthetic peptides. The synthetic, linear peptides that respond to the amino acid sequences of entire integrin-binding loops for obtustatin (CWKTSLTSHYC) and viperistatin (CWKTSRTSHYC) were tested in adhesion and ELISA assays. KTSR-containing peptide exposed IC<sub>50</sub> = 11.18  $\mu$ M (Fig. 2B) and 105.25  $\mu$ M (Fig. 4) in ELISA and adhesion assays, respectively, whereas KTSL-containing peptide showed IC<sub>50</sub> = 100.90 and 645.15  $\mu$ M in the same assays, respectively. As a control, we used synthetic peptide representing integrin binding-loop for echistatin. This peptide (CKRARGDDMDYYC) was neither active in ELISA nor in adhesion studies. Hence, replacement of a leucine residue (obtustatin) with an arginine (viperistatin) increases by 6- to 10-fold the inhibitory activity towards  $\alpha$ 1 $\beta$ 1. This conclusion is in line with previous studies showing the functional relevance of amino acids flanking both sides of the primary integrin binding motifs. Presence of tryptophane following RGD sequence in eristostatin potentially increased the activity of this disintegrin to inhibit  $\alpha$ Ib $\beta$ 3 integrin if compared with echistatin, containing aspartic acid in this site. On the other hand, echistatin blocked the function of two other RGD-dependent integrins,  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1, whereas eristostatin showed negligible ability to interact with these integrins.

Table 1  
Comparison of inhibitory effects of obtustatin, viperistatin and echistatin on various integrins in cell adhesion assay<sup>a</sup>

Cell suspension	Integrin	Ligand	IC <sub>50</sub> (nM)		
			Obtustatin	Viperistatin	Echistatin
$\alpha$ 1K562	$\alpha$ 1 $\beta$ 1	Coll IV	2	0.08	>10 000
$\alpha$ 1K562	$\alpha$ 1 $\beta$ 1	Coll I	0.5	0.02	>10 000
$\alpha$ 2K562	$\alpha$ 2 $\beta$ 1	Coll I	>10 000	>10 000	>10 000
$\alpha$ 2K562	$\alpha$ 2 $\beta$ 1	Coll IV	>10 000	>10 000	>10 000
$\alpha$ 6K562	$\alpha$ 6 $\beta$ 1	LM	>10 000	>10 000	>10 000
K562	$\alpha$ 5 $\beta$ 1	FN	>5000	>10 000	50
Jurkat	$\alpha$ 4 $\beta$ 1	VCAM-1	>10 000	>10 000	>10 000
SW480 $\alpha$ 9	$\alpha$ 9 $\beta$ 1	VCAM-1	>10 000	>10 000	>10 000
A5	$\alpha$ Ib $\beta$ 3	FG	>10 000	>10 000	50
JY	$\alpha$ v $\beta$ 3	VN	>10 000	>10 000	0.1

<sup>a</sup> The data represent means of the results of three experiments. Coll, collagen; LM, laminin; FN, fibronectin; VCAM-1, vascular cell adhesion molecule-1; FG, fibrinogen and VN, vitronectin.  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 6K562, K562 cells transfected with  $\alpha$ 1,  $\alpha$ 2 or  $\alpha$ 6 integrins; SW480 $\alpha$ 9 = SW480 cells transfected with  $\alpha$ 9 integrin; A5 = CHO cells transfected with  $\alpha$ Ib $\beta$ 3 integrin.

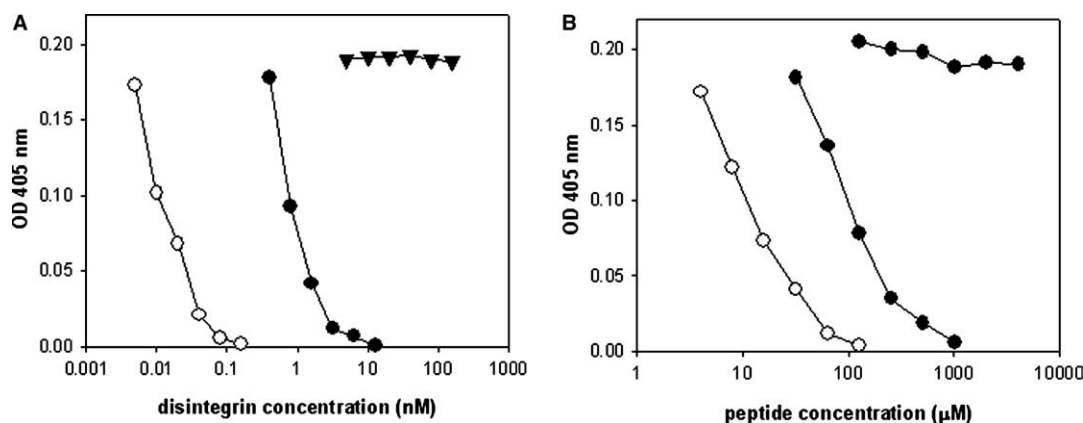


Fig. 2. Effect of increasing concentrations of disintegrins (A) and synthetic peptides (B) on the binding of collagen IV to immobilized, purified  $\alpha 1\beta 1$  integrin in ELISA. On the panel (A), filled circles represent obtustatin; open circles viperistatin; filled triangles echistatin. On the panel (B), filled circles represent obtustatin-derived peptide (CWKTSLSHTC); open circles viperistatin-derived peptide (CWKTSRTSHTC); filled triangles echistatin-derived peptide (CKRARGDDMDDYC). The data are represented by means calculated from three independent experiments.

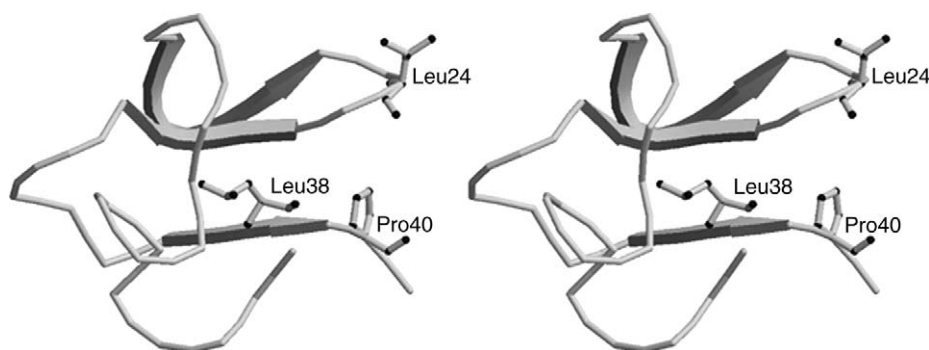


Fig. 3. Stereo drawing of the NMR solution structure of obtustatin (1MPZ) [22] showing the location of the three residues (rendered in the ball-and-stick representation) that differentiate this disintegrin from viperistatin: L<sup>24</sup>/R within the integrin binding loop, L<sup>38</sup>/V in the hydrophobic core, and P<sup>40</sup>/Q in the C-terminal region (Fig. 1).

Moreover, recombinant substitution of aspartic acid to tryptophane in echistatin molecule drastically decreased its activity to interact with  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  and increased potency to in-

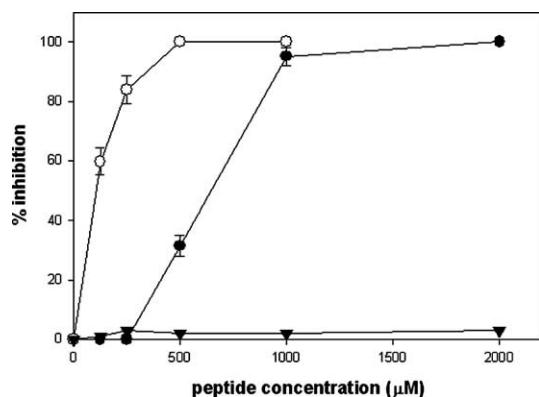


Fig. 4. Effect of synthetic peptides, designed on the basis of active site of obtustatin, viperistatin and echistatin in  $\alpha 1K562$  cell adhesion assay. The inhibitory effects of peptides CWKTSLSHTC (filled circles), CWKTSRTSHTC (open circles) and CKRARGDDMDDYC (filled triangles) were tested in an adhesion assay of CMFDA-labeled  $\alpha 1K562$  cells to immobilized collagen IV. The error bars represent the standard deviation in three independent experiments.

hibit  $\alpha 1\text{Ib}\beta 3$  [33]. Selectivity that is dependent from amino acid composition of integrin-binding loop has also been observed for MLD-containing disintegrins [21]. Threonine, which is adjacent to MLD motif from N-terminus significantly, increased activity of VLO5 to block human  $\alpha 4\beta 1$  and  $\alpha 9\beta 1$  integrins in comparison with EC3 that contains alanine in this place.

The partial relative enhancement of the inhibitory activity of the KTSR-peptide suggests that the L<sup>38</sup>/V and P<sup>40</sup>/Q substitutions of viperistatin may also contribute to the biological activity of this disintegrin. The L<sup>38</sup>/V substitution within the hydrophobic core of viperistatin may not significantly alter the conformation of the disintegrin, and may therefore represent a neutral mutation. On the other hand, NMR studies of obtustatin [22,29] showed that the integrin binding loop and the C-terminal tail are structurally linked and display concerted motions in the 100–300 ps time-scale, strongly indicating that these two functional regions may form a conformational epitope (Fig. 3) engaged in extensive interactions with the target integrin receptor. The P<sup>40</sup>/Q substitution, along with the active site L<sup>24</sup>/R mutation, may therefore create a distinct chemical environment responsible for the increased inhibitory activity of viperistatin over obtustatin.

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